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Original article:

ON-SITE CELLULASE PRODUCTION BY *TRICHODERMA REESEI* $3EMS_{35}$ MUTANT AND SAME VESSEL SACCHARIFICATION AND FERMENTATION OF ACID TREATED WHEAT STRAW FOR ETHANOL PRODUCTION

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ABSTRACT

Bioethanol production from lignocellulosic raw materials involves process steps like pre-treatment, enzymatic hydrolysis, fermentation and distillation. In this study, wheat straw was explored as feedstock for on-site cellulase production by *T. reesei* $3EMS_{35}$ mutant, and as a substrate for second generation bioethanol production from baker yeast. Scanning electron microscopy (SEM) and X-ray diffractography (XRD) of untreated wheat straw (UWS) and acid treated wheat straw (TWS) were done to understand the structural organization and changes in the cellulase accessibility and reactivity. The effect of delignification and structural modification for on-site cellulase enzyme production was comparably studied. The efficiency of crude cellulase enzyme for digestion of UWS and TWS and then production of ethanol from TWS was studied using same-vessel saccharification and fermentation (SVSF) technique, both in shaking flasks as well as in fermenters. Two different methods of operation were tested, i.e. the UWS_{Enz} method, where UWS was used for on-site enzyme production, and TWS_{Enz} method where TWS was applied as substrate for cellullase production. Results obtained showed structural modifications in cellulose of TWS due to delignification, removal of wax and change of crystallinity. UWS was better substrate than TWS for cellulase production due to the fact that lignin did not hinder the enzyme production by fungus but acted as a booster. On-site cellulase enzyme produced by *T. reesei* $3EMS_{35}$ mutant hydrolyzed most of cellulose (91 %) in TWS within first 24 hrs. Shake flasks experiments showed that ethanol titers and yields with UWS_{Enz} were 2.9 times higher compared to those obtained with TWS_{Enz} method respectively. Comparatively, titer of ethanol in shake flask experiments was 10 % higher than this obtained in 3 L fermenter with UWS_{Enz}. Outcomes from this investigation clearly demonstrated the potential of on-site cellulase enzyme production and SVSF for ethanol production from wheat straw.

Keywords: on-site cellulase production, bioethanol, *S. cerevisiae*, *T. reesei*, SVSF, wheat straw, enzymatic hydrolysis

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INTRODUCTION

Wheat straw is a byproduct of wheat crop (*Triticum aestivum L.*) which is cultivated throughout the world. Small amount of wheat straw (25 %) is used as livestock feed and domestic fuel in developing countries, while a large part of it is discarded on the field or burned directly, which results in the waste of fuel resource as well as environmental contamination (Atwell, 2001). Wheat straw does not compete with primary food products and due to its wide availability and low cost, it has highest bioenergy potential among all agricultural residues (Erdei et al., 2010).

The production of bioethanol from wheat straw involves four basic steps; pretreatment, hydrolysis, fermentation and recovery of ethanol. At present main problem is that all these laborious steps/processes are being done separately using more time, instrumentation, manpower and high costs for purchase of commercial enzymes.

Many pretreatment methods have been developed for delignification of wheat straw and overall efficiency of any method depends on balance between low inhibitors formation and high hydrolysis (Demirbas, 2005). According to Saliu and Sani (2012) pretreatment of biomass plays a very important role in process of production of ethanol and its objectives are to increase the total available surface area and porosity of the substrate, to reduce the crystallinity of cellulose fibers to make it more fibrillated and disrupt the heterogeneous structure of cellulosic materials. Among various pretreatment methods, thermo-chemical method such as dilute acid pretreatment is most effective because it removes wax and lignin more effectively (Kristensen et al., 2008; Barta et al., 2010; Idrees et al., 2013). Scanning electron microscopy (SEM) is a widely used technique to explain the structural changes before and after pretreatment of lignocellulosic biomass. X-ray diffractography (XRD) diffraction explains well the crystallinity of the biomass (Xiao et al., 2011). The degree of crystallinity and modification in structure of cellulose are two well established factors for determination and calculation of efficiency of enzymatic hydrolysis of biomass (Çetinkol et al., 2010). SEM images and XRD diffractogram can be used to understand to what extent a specific pretreatment method is successful for reduction of lignin content in lignocelluloses and for alteration of the lignocellulosic structure (Wang et al., 2013). Contrary to the conventional methods for analysis of lignocellulosic material, XRD has advantages of short analysis time, small samples and less destruction.

Enzymatic hydrolysis of cellulose is a promising method for the conversion of waste lignocellulose to fermentable sugars. Many factors, such as the content of lignin, crystallinity and degree of polymerization (DP) of cellulose, moisture content and particle size, affect the digestibility of the cellulose present in the biomass (Mehmood et al., 2009; Kim and Lee, 2006). On-site or near-site production of cellulase on cheap lignocellulosic biomass (for example, wheat straw) has been investigated in different studies (Sorensen et al., 2011). It is a better technique for reduction of the process cost instead of utilization of expensive commercial enzymes purchased from enzyme manufacturers. However, to our knowledge so far, integration of on-site cellulose production with same vessel hydrolysis and fermentation (SVSF) has not been studied. The cellulolytic fungus *T. reesei* looks promising for on-site cellulase production due to its superior features, i.e., capability to produce all components of cellulase complex; endocellulase, exocellulase and β-glucosidase in good proportions as well as production of other enzymes such as xylanases or laccases (Arantes and Saddler, 2010) in comparison to other enzyme producers. Therefore, the aim of the present study was to investigate the potential of treated and untreated wheat straw as feedstock for on-site cellulase production by a fungus *T. reesei* $3EMS_{35}$ and ethanol production by the yeast *S. cerevisiae*. SEM and

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XRD were applied to characterize and gain insight on delignification process, structural modification and change in crystallinity of cellulose in wheat straw. Process of hydrolysis of cellulose into glucose and fermentation of glucose into ethanol were integrated to cut down process cost and time. The technique was named as same vessel saccharification and fermentation, abbreviated as SVSF (Karagöz et al., 2012).

MATERIALS AND METHODS

Microorganisms

Yeast strain *Saccharomyces cerevisiae* PCSIR-12 was maintained on yeast media slants containing (g/l): dextrose 10, peptone 5, yeast extract 3, malt extract 3 and agar 20 (Gaensly et al., 2011) at 4 °C and transferred every 6 weeks. The fungus *T. reesei* $3EMS_{35}$ developed by multi-agent mutagenesis was maintained on the PDA slants at 4 °C and was subcultured monthly.

Preparation of inocula

25 ml synthetic yeast medium containing (g/l): dextrose 10, peptone 5, yeast extract 3, malt extract 3 (Gaensly et al., 2011) was dispensed in 250 ml conical flasks, sterilized and inoculated with one loop of *S. cerevisiae*-PCSIR-12 from 24 hr old culture grown in the yeast medium agar slants and incubated at 28 °C, 120 rpm for 24 hrs.

Cellulase production in submerged fermentation

4.0 g wheat straw already pretreated (TWS) by dilute sulfuric acid as reported by Khokhar et al. (2010) was taken in a 500 ml conical flask. 100 ml of 1x vogal's medium and 0.2 % (w/v) galactose was added (modified Vogel's medium). pH was adjusted to 6.0 with 10 % NaOH and sterilized for 20 min. Inoculation was made with 72 hour old, 2 ml spore suspension ($6.05x10^6$ spores/ml) of *T. reesei* $3EMS_{35}$ mutant. Flasks were incubated for 72 hrs at 30 °C and 140 rpm on shaker (IKA® KS 4000 i control) for cellulase production.

Cellulase production in solid state fermentation

4.0 g TWS/UWS was taken in 500 ml conical flask and wet with 10-15 ml modified vogal's medium. Flask was sterilized for 15 min and then inoculated with 2.0 ml conidial suspension of 72 hr old *T. reesei* $3EMS_{35}$ mutant and mixed well for 2 min. It was incubated for 72 hrs at 30 °C and 150 rpm. Then 100 ml distilled water and 0.1 g Tween 80 was added. Hydrolysis and fermentation steps were carried out as below.

On-site cellulase production and same vessel saccharification and fermentation (SVSF)

After production of cellulose enzyme in submerged fermentation as described above, 0.1 g Tween 80, was added to facilitate hydrolysis (Mehmood et al., 2009), and temperature was increased to 50 °C for saccharification. Small samples were removed after regular intervals for HPLC analysis of reducing sugars.

The flasks were allowed to cool down at room temperature and yeast nutrients (g/l) were added: peptone 5, yeast extract 3, malt extract 3 and $MgSO_4$. Then 5 %, 24 hr old culture of *S. cerevisiae* (3×10^7 spores/ml) prepared in defined yeast medium (Mehmood et al., 2009) was added as inoculum in the flask for ethanol production. Flasks were incubated at 37 °C, with shaking at 120 rpm. Samples were removed from fermentation vessels at regular intervals of time to monitor the time course for ethanol and residual sugars.

Initial concentration of substrate plays a critical role in ethanol production. In this study 1.0, 2.0, 3.0, 4.0, 5.0 and 6.0 % concentration of wheat straw was tested in SVSF for ethanol production.

Different techniques (Table 1) were tested for on-site enzyme production and then SVSF for ethanol production i. e. (I) Submerged Fermentation: TWS_{Sub} and UWS_{Sub}; 5 g treated wheat straw and untreated wheat straw in 100 ml modified Vo-

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gel medium was used for enzyme production and SVSF for ethanol production, this technique we called as submerged fermentation. (II) Solid Substrate Fermentation: TWS_{Solid} and UWS_{Solid}; 5 g treated wheat straw and untreated wheat straw and only 12-15 ml of modified Vogel medium, just to wet the substrate, was taken in flask for enzyme production. (III) UWS_{Enz}; 1 g untreated wheat straw, in 100 ml modified Vogel medium, was used for enzyme production which was then used for hydrolysis and fermentation of 4 g treated wheat straw in SVSF. This method was represented as UWS_{Enz}-$TWS_{Enz.}$ (IV) TWS_{Enz}; 1 g treated wheat straw, in 100 ml modified Vogel's medium, was first used for enzyme production which was then used for hydrolysis and fermentation of 4 g treated wheat straw in SVSF. This method was represented as TWS_{Enz}-TWS_{Enz} (Table 1).

SVSF for ethanol production in the fermenter

SVSF experiments were also conducted in 3 litre fermenter with 1 litre working volume. 5 g UWS, 500 ml 1x modified Vogel's medium and 2 g galactose were dispensed in the fermentation tank of fermenter [BYWS (Shanghai) Water Treatment Technology Co. Ltd] Model AWTR3L-01-V1.0. It was sterilized in the automatic sterilizer (*Systec* V-65, Holm and Halby) for 20 min at 121 °C, allowed to cool at room temperature and then 1 ml vitamin solution was added. Vitamin solution was not autoclaved not to deactivate the vitamins (Angelidaki and Sanders, 2004). Fermentation tank was fitted on the control unit of fermenter. Control unit was set to automatically adjust the pH 6, temperature 30 °C, 140 rpm. 25 ml vegetative spores of 72 hr old *T. reesei* $3EMS_{35}$ mutant were used as inoculum. After 72 hr of fermentation for cellulase enzyme production, 30 g separately autoclaved TWS in 500 1x Vogel's medium was added in the fermenter. Then 1.0 g Tween 80 was also added and temperature was raised to 50 °C for hydrolysis/saccharification of TWS. Samples were taken out after regular intervals of time. Then temperature 37 °C, pH 5.5 was adjusted and yeast nutrients were added. 50 ml of 24 hr old culture of *S. cerevisiae* PCSIR-12 was inoculated and N_2 gas was sparged for 10 min to create anaerobic conditions. During fermentation for ethanol production, samples were taken after regular interval for HPLC analysis.

Table 1: Different modes of on-site cellulase production and SVSF

On-site cellulase enzyme production					same vessel saccharification and fermentation (SVSF)
Modes of Enzyme production	Biomass		1x Vogel's medium used (ml)		Amount of biomass used for SVSF/(g)
	UWS (g)	TWS (g)	100	12-15	
TWS_{sub}	0	5	+	-	TWS/0
UWS_{sub}	5	0	+	-	UWS/0
TWS_{solid}	0	5	-	+	TWS/0
UWS_{solid}	5	0	-	+	UWS/0
UWS_{enz}	1	0	+	-	TWS/4
TWS_{enz}	0	1	+	-	TWS/4

Note: + = yes, - = no

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Toxicity/adaptivity test for maximum ethanol production by Saccharomyces cerevisiae-PCSIR-12

The hydrolysate of lignocellulosic biomass of wheat straw produced using on-site produced cellulase, may contain some by-products toxic for *S. cerevisiae*. So toxicity/adaptivity tests were performed. Hydrolysate was diluted with synthetic yeast medium and fermented by *S. cerevisiae*-PCSIR-12 under optimum conditions. The dilution/concentration which resulted to highest ethanol production was selected as optimum, less toxic for *S. cerevisiae*-PCSIR-12 (Table 2, Figure 6).

Scanning electron microscopy (SEM)

Samples of wheat straw were mounted on the stubs and sputter-coated (sputter Emitech/ K550 model) with Au prior to imaging with FEI QUANTA 200 FEG Scanning electron microscopy (SEM) using 20 KV accelerating voltage and working distance 38 mm. Many SEM images were obtained with different magnifications (Angelidaki et al., 2009).

X-Ray diffraction chromatography (XRD)

X-ray powder diffraction patterns were collected in θ-θ reflection mode on a Bruker D8 Advance diffractometer using CuKα radiation, a secondary graphite monochromator and an automatic divergence slit. Data were collected in the 2θ-range 5–60° with a step size of 0.02° and a counting time of 20 s per step. 2θ is an important parameter showing the diffraction angle of X-rays due to interaction with the surface of biomas (Li et al., 2010). XRD data was used for calculation of crystallinity index (*CrI*) using formula of Segal et al. (1959) as follows:

$$CrI = [(I_{002} - I_{am})/I_{002}] \times 100$$

where I_{002} is the intensity for the crystalline portion of biomass i.e. cellulose at about 2θ = 22.5, and I_{am} is the peak for amorphous portion (cellulose, hemicellulose and lignin) at about 2θ = 18.7 in the literature (Li et al., 2010).

High performance liquid chromatography (HPLC) analysis

Products of hydrolysis and fermentation, sugar monomers and ethanol, were measured using HPLC (Agilent). BioRad, Aminex HPX-87 H column (300 x 7.8 mm) was used with 0.04 M sulfuric acid eluent, with flow rate of 0.6 ml/min and oven temperature at 65 °C. Refractive Index detector was used for sugars, acetic acid and ethanol and a UV detector at 280 nm was used for furfural and HMF (Karagöz et al., 2012; Angelidaki et al., 2009).

RESULTS AND DISCUSSION

Effect of pretreatment on cellulose crystallinity

Scanning electron microscopy was used to explore the modifications in wheat straw fibers due to hammer beater mill grinding and then acid pretreatment. Significant changes in the morphology and texture of wheat straw after pretreatment were observed (Figure 1). Untreated wheat straw is composed of lignin, cellulose and hemicelluloses hence called lignocellulose in which cellulose is tightly bound by lignin and hemicelluloses. Lignin is the cementing agent and makes the cellulose hard and crystalline. Surface of untreated wheat straw was smooth, nonporous and more compact (Figure 1). Cellulosic fibers were not visible even at very high magnification in SEM image C (10000 x Magnified). After pre-treatment (Figure 1D, E, F) lignin and hemicellose has been removed to great extent and texture was changed. Apparent changes on the surface were seen including ridges, scars and cracks, disappearance of the cohesion within the fibers and exposure of internal cell wall. Intact cellulose fibers were clearly visible even at comparatively less magnification. Results obtained here clearly demonstrated that after acid pretreatment cellulose became naked, less crystalline and more accessible for cellulase

enzyme. Our findings are in agreement with investigations of Wang et al. (2013) who reported that defibrillation and delignification released a large reactive area on the fiber surface, consequently accessibility of cellulose and bioconversion efficiency was improved. Our investigations are in contrary to results reported by other investigators (Kristensen et al., 2008; Xiao et al., 2011) where no globular granules were seen on the cellulosic fiber of acid pretreated wheat straw; the possible reason may be that dilute acid dissolved and removed lignin and wax more effectively than hydrothermal pretreatment. Hammer beater mill grinding and dilute acid pretreatment combinely loosen the cellulose fibers in the cell wall and created fibrillation (Figure 1E and 1F) that increased micro fibril exposure to cellulase enzyme (Tao et al., 2012). It was concluded that diluted acid pretreatment is better than any other type of pretreatment of wheat straw due to two reasons: (1) Dilute acid removes wax, lignin and hemicelluloses. (2) Cellulose fibers are not ruptured, broken or destroyed but remain intact and can be seen at very low magnification of SEM image (Figure 1F). Most important factors for hydrolysis are the physical state and structure of the substrate (Angelidaki and Sanders, 2004; Chartchalerm et al., 2007) and enzymatic hydrolysis of cellulose is a surface phenomenon, if cellulose is free (pretreated) hydrolysis is high and if cellulose is entrapped into the lignin complex then its hydrolysis will be very low (Tong et al., 1990).

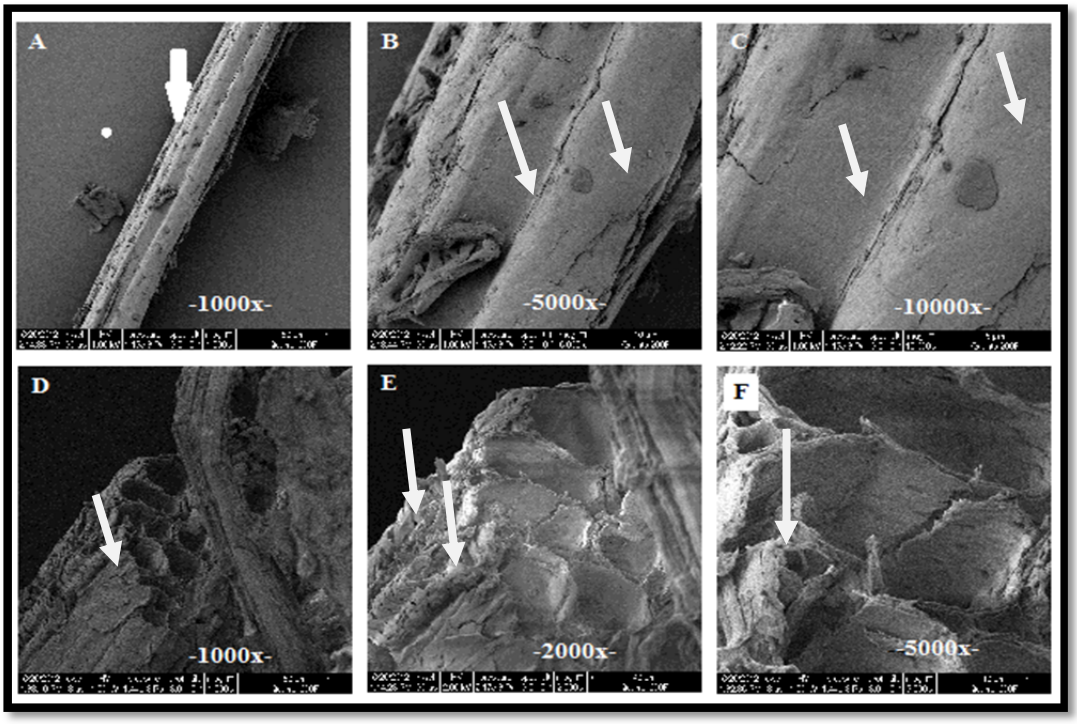

Figure 1: Scanning electron microscopic (SEM) images of untreated wheat straw (control) are shown (**A-C**) and dilute H_2SO_4 pretreated wheat straw by (**D-F**).The surface of untreated wheat straw is surrounded by a sheath leaf and is looking very smooth and brighter due to presence of lignin (A, SEM image 1000X magnified). B and C are SEM images of same straw at higher magnification (B 5000x and C 10000x) even then surface is looking smooth, compact and rigid. But in the SEM images of 1.5 % H_2SO_4 treated wheat straw (D, E and F) surface of treated wheat straw is rough, soft and faint due to removal or re-location of lignin. Yet D, E and F are less magnified (1000x, 2000x and 5000x) than (A, B and C) even then the straw cavities show clearly interwoven cellulose microfibrils. In dilute acid pretreatment wax is washed, lignin is partially removed and individual fibers are separated. E and F are more magnified SEM images (2000x and 5000x) and clearly show that delignification exposes intact, interwoven cellulose microfibrils and cellulose lamellae or agglomerates become prominent.

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Table 2: Adaptivity tests of *S. cerevisiae* $3EMS_{35}$ for various dilutions of hydrolysate for bioethanol production in SVSF process

Hydrolysate from submerged fermentation					Hydrolysate from solid substrate fermentation				
Concentration of hydrolysate (%)	Glucose (g/l)	Xylose (g/l)	Citrate (g/l)	Ethanol (g/l)	Concentration of hydrolysate (%)	Glucose (g/l)	Xylose (g/l)	Citrate (g/l)	Ethanol (g/l)
20	0.00	0.6	1.07	2.50	20	0.00	0.7	0.46	2.27
40	0.00	1.2	1.65	2.62	40	0.00	0.9	0.73	2.70
60	0.03	1.3	1.68	2.67	60	0.06	1.2	0.75	2.51
80	0.09	2.1	3.09	3.85	80	0.07	1.9	1.25	2.62
100	0.08	2.4	2.50	4.56	100	0.09	2.6	1.46	3.94

To better understand the physical and chemical changes/alterations in the structure and texture of the wheat straw, X-Ray diffractography of UWS, TWS and residue left after hydrolysis and fermentation of wheat straw in SVSF process was done. The X-Ray diffractogram showed that intensity of X-rays for untreated wheat straw (UWS) was higher compared to the treated wheat straw (TWS), demonstrating that the presence of lignin and rigidity of the biomass was higher in the UWS (Figure 2). Dilute acid successfully removed lignin and wax, consequently, crystallinity of acid pretreated biomass (TWS) was decreased and the intensity. XRD diffractogram (Figure 2) showed great difference in intensity of X-rays absorbed by untreated and treated wheat straw indicating that acidic pretreatment removed the lignin, hemicellulose and wax consequently crystallinity of TWS is decreased but fibril coherence and alignment were largely retained (Ibbett et al., 2013). Evidence from XRD data indicated that after acid pretreatment crystallinity index (*CrI)* was decreased by 12.5 %. *CrI* of acid treated wheat straw remained same (45.12) before and after enzymatic hydrolysis and fermentation in SVSF and peaks for TWS and H&F wheat straw were identical (Figure 2) showing that degree of crystallinity of biomass was not affected during the hydrolysis and fermentation steps. In this study two peaks were achieved; 2θ = 22.5 which corresponds to the crystalline region of biomass i. e. cellulose only, and 2θ = 16.4 which corresponds to the amorphous region of biomass, i. e. cellulose, hemicellulose and lignin (Li et al., 2010). Acidic pretreatment is more effective for delignification compared to biological (fungal) treatment (Saha et al., 2005) due to slower delignification rate during biological treatment. Wang et al. (2013) compared XRD and fourier transform infrared (FTIR) data for untreated and biologically (white rot fungus) pretreated poplar chips and reported that crystallinity was not changed in either case.

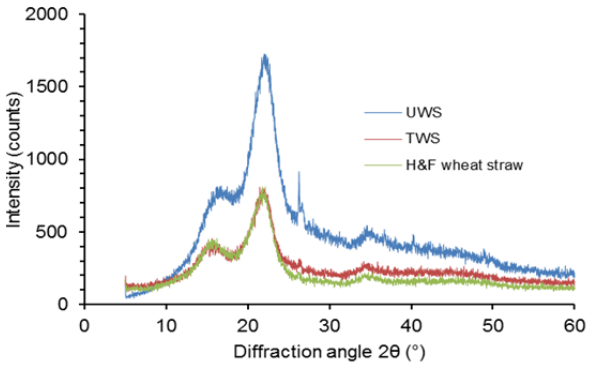

Figure 2: X-Ray diffractogram of UWS, TWS and residue left after hydrolysis and fermentation (H&F) of wheat straw in SVSF process. XRD was kindly done by Kenny Stahl, Chemistry Department, Technical University of Denmark. XRD data obtained was used for calculation of crystallinity index (*CrI*).

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Shake flasks experiments

On-site production of cellulase

CMCase specific activity of cellulase enzyme produced by *T. reesei* $3EMS_{35}$ on UWS was already shown to be 2.4-fold higher than specific activity when TWS was used. Similarly, specific FPase activity of cellulase produced on UWS was 1.3-fold higher than that produced on TWS, respectively. During enzyme production, the content in the vessels containing UWS showed very good growth of *T. reesei* $3EMS_{35}$ and some spores/mycelia were attached to walls of flask and also floating on the liquid surface (data not shown), indicating that growth of the *T. reesei* $3EMS_{35}$ was more prominent as compared to vessel containing TWS. The above finding indicated that UWS was better substrate for cellulase enzyme production than TWS. A possible explanation for this could be that in case of UWS, cellulose is tightly bound and thereby less available for microbial utilization. As a result, fungal cells secrete more cellulase enzyme to digest cellulose into glucose (Cullen and Kersten, 1996). Lignin does not inhibit but promotes production of cellulase enzyme of high specific activity (Reczey et al., 1996), indicating that delignified plant biomass is not good substrate compared to non-delignified plant biomass. Previous studies also showed that lignocellulases (cellulases) are co-related to the stationary phase of growth of fungi and their production was triggered by nutrients limitation in the medium (More et al., 2011). *T. reesei* $3EMS_{35}$ produced the cellulase complex in proper proportion. This cellulase complex hydrolysed cellulose of wheat straw successfully into fermentable sugars. Note that all fungal strains, other than *T. reesei* $3EMS_{35}$, cannot produce all the components of the cellulose complex (endocellulase, exocellulase and β-glucosidase) in proper proportion and such cellulase enzymes fail to hydrolyze lignocellulose in to fermentable sugars completely. In this situation, addition of one or more commercial enzymes is required (Hu et al., 2013). For example, Sorensen et al. (2011) produced on-site cellulase enzyme by *A. niger* fungus on the filter cake left after hydrolysis and fermentation of wheat straw. They found that cellulase enzyme of *A. niger* alone was unable to hydrolyze the TWS but it needed addition of the commercial enzyme, celluclast, for hydrolysis and fermentation of pretreated biomass. In case of on-site production, microorganisms may produce mixture of endocellulase, exocellulase (Wang et al., 2013) and β-glucosidase in good proportion and also other enzymes like xylanases or laccases may be produced in small quantity depending upon the composition of substrate. Our findings are in agreement with Arantes and Saddler (2010) who reported that cellulose-degrading microorganisms also produce some accessory proteins that are co-regulated and co-expressed with the cellulase enzymes. These auxiliary proteins do not hydrolyze cellulosic material *per se*, but play a significant role in enhancing the yield by increasing the access of cellulases to the substrate and opening the crystalline structure (Hu et al., 2013). Such enzymes are the swollenins and expansins. On-site produced cellulase enzymes have advantages over commerial enzyme mixtures which produce some unwanted byproducts, like gluconic acid and cellobionic acid from lignocelluloses (Cannella et al., 2012), thereby preference of on-site produced cellulase enzyme over commercial enzyme mix was suggested.

Saccharification/hydrolysis of acid treated wheat straw (TWS)

Rate of hydrolysis of lignocellulose is very important with respect to economics of bioethanol production technology. It depends on nature of lignocellulose, method of pretreatment and mixture of cellulases. The crude cellulase enzyme containing other proteins, fungal mycelia debris, without any purification was used for hydrolysis of lignocellulose of wheat straw to glucose. Results obtained (Figure 3) showed that most of the cellulose was hydrolyzed rapid-

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ly within 24 hrs, whereafter the rate of hydrolysis was slowed down. These results are fully in agreement with other studies (Luo et al., 2011; Kumar et al., 2009) where commercial cellulase enzyme was used for saccharification of pretreated rapeseed straw. On-site produced cellulase enzyme employed in our study was superior and resulted in faster hydrolysis than reported by some other investigations (Mehmood et al., 2009; Han et al., 2012; Li et al., 2010). The difference in the hydrolysis could be explained with the different nature of biomass used in our study compared to the others. Rate of hydrolysis and products obtained are affected by pretreatment methods, physiochemical structural and compositional factors (Kumar et al., 2009). The rate of saccharification in our study was 8 % lower than reported in Tao et al. (2012), that by deacetylation and mechanical refining of acid pretreated corn stover, enzymatic saccharification was boosted up to 98 %. Most probably, this difference in rate of hydrolysis was due to deacetylation and mechanical refining of the biomass.

Optimization of initial concentration of wheat straw as a substrate for hydrolysis and ethanol production in SVSF was also investigated using 1, 2, 3, 4, 5, 7, 10 % (w/v) wheat straw. 4.0 % TWS was found to be the optimum concentration of substrate for enzymatic hydrolysis and ethanol production (Figure 4). 91 % cellulose was hydrolyzed into glucose in 4 % TWS within 24 hrs, at 50 °C. Our results are in agreement with investigation of Luo et al. (2011) and also Socha et al. (2013) who reported 70-92 % glucose yield after enzymatic hydrolysis, using commercial enzymes, of ionic liquid pretreated residue of Douglas-fir and forestry wood wastes.

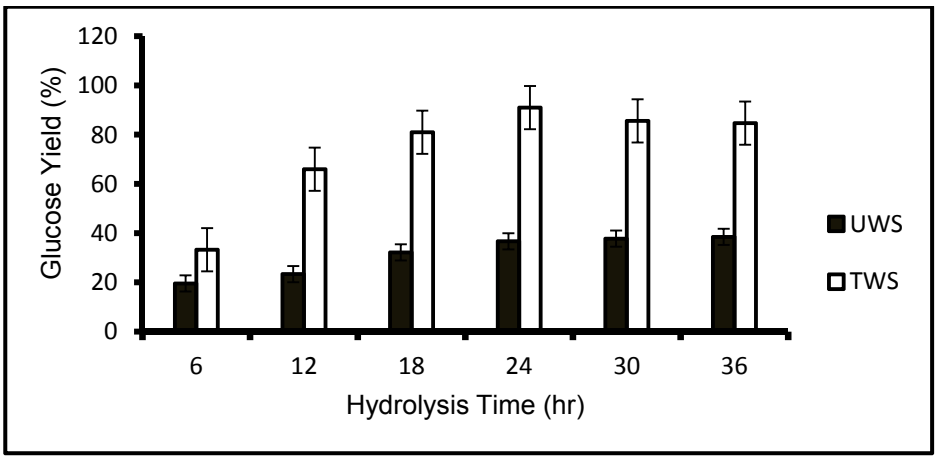

Figure 3: Optimization of time for hydrolysis of wheat straw (TWS and UWS) by on-site produced cellulase enzyme. Crude cellulase enzyme along with fungal mycelia was used for hydrolysis of TWS and UWS at 50 °C on orbital shaker.

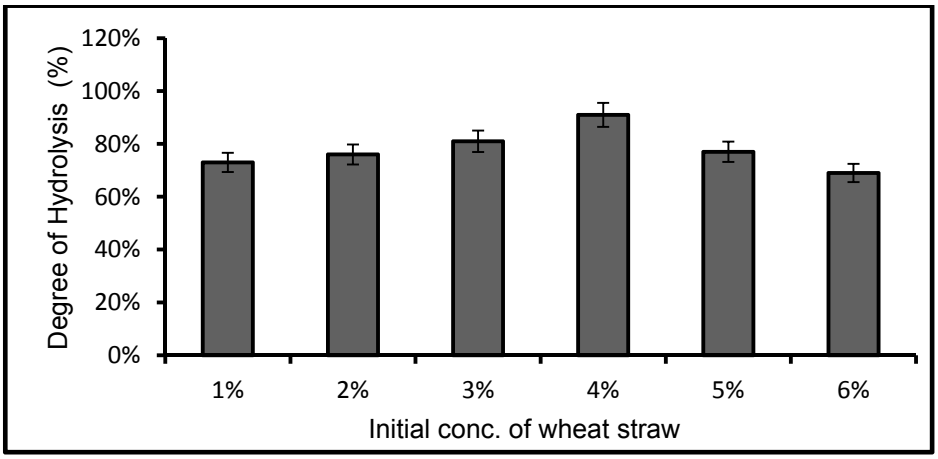

Figure 4: Initial concentration of acid treated wheat straw was optimized for hydrolysis by on-site produced cellulase enzyme by *T. reesei* $3EMS_{35}$ mutant. The optimum concentration for maximum hydrolysis (91 %) in 24 hr was 4 % (40 g TWS/l).

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Fermentation by Saccharomyces cerevisiae PCSIR-12

Ethanol is produced by a fermentation process, in which microorganisms convert the fermentable carbohydrates, e.g. maltose, glucose and xylose into ethanol and resulting in a fermentation broth of complex mixture of living yeast cells, nutrients, fungus cell debris and other products/byproducts of the fermentation process. It is generally known that the ethanol concentration is inversely proportional to the concentration of carbohydrates. Therefore, monitoring of carbohydrate levels serves as a key indicator in determining when to stop the reaction. Results obtained (Figure 5a) showed that with the fermentation time up to 48 hrs, the concentration of glucose was decreased gradually from 1.5 to 0.05 (g/l), while the yield of ethanol was increased respectively from 17 % to 48 % of theoretical yield. The highest ethanol yield (48 %) equivalent to the concentration of 9.02 g/l (Figure 5b) in the fermentation broth was reached after 48 hrs of SVSF from acid treated wheat straw (TWS).

On-site cellulase enzyme production and SVSF were integrated to evaluate the ethanol production from TWS. The integration of process stages has several advantages including: no cast of cellulase, less instrumentation, less time and good ethanol yield. The ethanol yield from TWS (48 %) was not as good as reported in the literature (Zhua et al., 2006; Chen et al., 2008; Erdei et al., 2010; Luo et al., 2011) perhaps due less production of cellulase enzyme, of low specific enzyme activity, on the TWS.

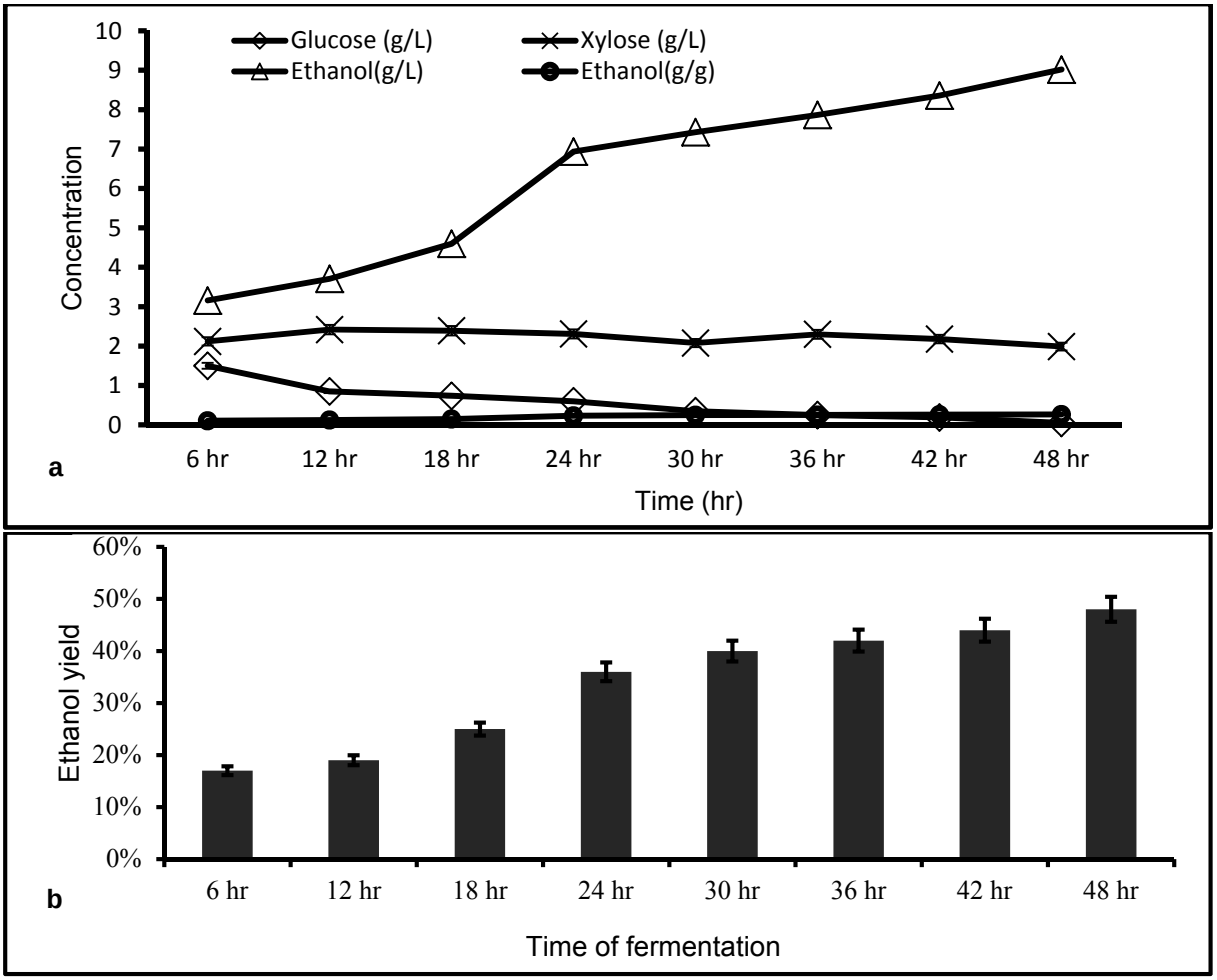

Figure 5: On-site cellulase production by *T. reesei* $3EMS_{35}$ mutant and SVSF: (**a**) Rate of ethanol production by *S. cerevisiae,* in SVSF. Ethanol production is shown as (g/l) and (g/g) of TWS; (**b**) Yield (%) of ethanol in SVSF of theoretical yield from UWS

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Adaptivity/toxicity test

As all the steps: (I) production of cellulase enzyme, (II) hydrolysis of pretreated wheat straw into glucose, and (III) fermentation of glucose into ethanol, were carried out in the same vessel, there may be some toxic compounds produced, which may show some adverse or toxic effects to the *S. cerevisiae* (Detroy et al., 1982). To check the toxicity of byproducts, adaptivity tests were conducted, in which hydrolysate was diluted to 20, 40, 60, 80 and 100 % (pure hydrolysate) and subjected to anaerobic fermentation using *S. cerevisiae* PCSIR-12. The result obtained demonstrated highest production of ethanol in the 100 % hydrolysate (Table 2 and Figure 6). It shows that no toxic byproducts, weak organic acids, phenolic compounds, furfural or hydroxylmethyl furfural (HMF) were present in the hydrolysate and no toxic effect on the *S. cerevisiae* PCSIR-12 was noted. Toxic compounds produced by pretreatment and hydrolysis reduce the ethanol yield (Detroy et al., 1982). Our findings are in agreement to previous literature (Saha et al., 2005) that by sulfuric acid pretreatment of rice hulls at temperature below 180 °C and subsequent enzymatic saccharification no toxic compounds were produced.

It also support the SEM images (Figure 1) that dilute sulfuric acid pretreatment was successful to remove wax and lignin, most of the lignin was removed and no furfural and HMF were present in the hydrolysate. A comparative study of Table 2 shows that using TWS as carbon source, submerged fermentation is better than solid substrate fermentation. Ethanol production in the case when cellulase was produced in submerged fermentation and used in SVSF of TWS is higher in each dilution level (20, 40, 60, 80 and 100 %) as compared to the case when cellulase was produced in solid substrate fermentation and then used for SVSF of TWS. For 100 % hydrolysate, ethanol yield was 4.56 g/l in case of submerged fermentation and 3.94 g/l in case of solid substrate fermentation of TWS. Comparatively 16 % more ethanol was produced in case of submerged fermentation than solid substrate fermentation of TWS. So in next of experiments 100 % concentrated hydrolysate was used for ethanol production by *S. cerevisiae*.

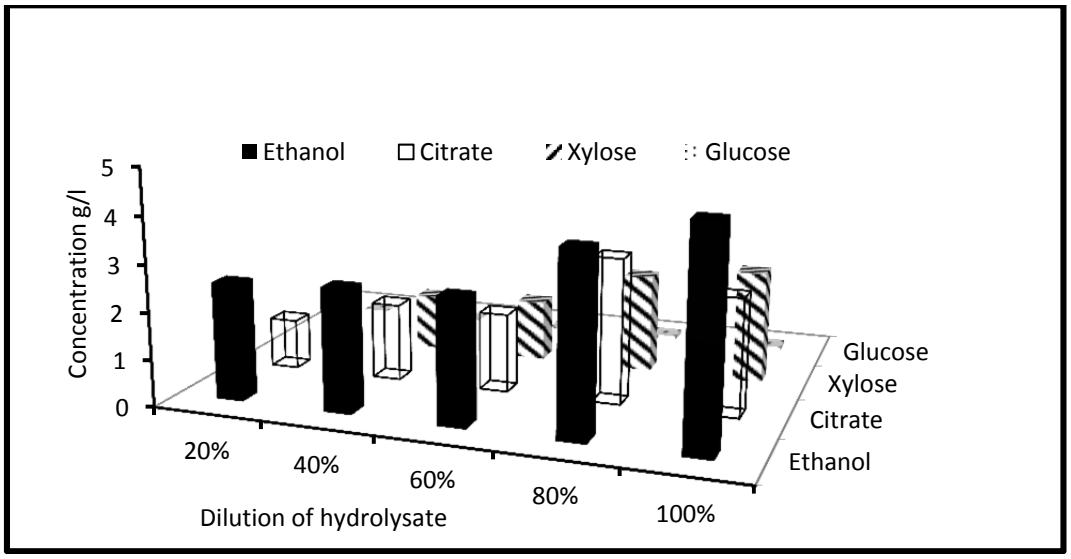

Figure 6: HPLC results of adaptivity test of *S. cerevisiae* for hydrolysate produced by submerged fermentation of wheat straw for maximum production of bioethanol

Note: Suitability of the composition of hydrolysate for growth of *S. cerevisiae* was checked by diluting to the level of 20, 40, 60, 80, and 100 % for ethanol production.

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Submerged versus solid substrate fermentation for bioethanol production

Submerged and solid state fermentation were evaluated in this study for ethanol production from wheat straw. Results obtained (Figure 7) clearly demonstrated that in case of submerged fermentation 71 % more ethanol was produced by TWS (TW_{Sub}) as compared to (UWS_{Sub}) respectively. Figure 7 also presents the comparison of on-site production of cellulase enzyme in solid substrate fermentation, using treated wheat straw (TWS_{solid}) and untreated wheat straw (UWS_{solid}) respectively. Cellulase enzyme was subsequently utilized saccharification of cellulose to glucose which in turn used in the SVSF for ethanol production. This comparative study showed that TWS_{solid} was superior to UWS_{solid} for ethanol production after 48 hr fermentation with *S. cerevisiae*. Ethanol yield was 0.06 and 0.04 g/g DM respectively (Figure 7).

It was concluded that UWS was best for cellulase enzyme production by *T. reesei* $3EMS_{35}$ mutant and not for ethanol production, while TWS was not good for enzyme production but best for saccharification by cellulase and fermentation to ethanol production in SVSF process. Sorensen et al. (2011) reported that filter cake (FC) left after hydrolysis and fermentation of TWS proved to be best carbon source for on-site production of cellulase enzyme by *A. niger*. Actually FC was that wheat straw which was not pretreated very well and lignin was left in it. So it was not hydrolyzed and fermented very well but proved good substrate for on-site cellulase enzyme production. Therefore in another batch, we tested a new technique, represented as UWS_{Enz}-TWS_F, in which 1 % UWS was first used for enzyme production. This enzyme along with fungal cells and substrate residue was used for saccharification of 4 % autoclaved TWS (total working volume 100 ml) and then fermentation with *S. cerevisiae* for ethanol production. For comparison, 1 % TWS for enzyme production and 4 % TWS for saccharification and fermentation for ethanol production was also tested in another flask. This technique was represented as TWS_{Enz}-TWS_F . The ethanol production was 13.5 g/l or 0.134 g/g DM of untreated wheat straw in the combined UWS_{Enz}-TWS_F method and the yield was 72.0 % of the theoretical yield (Figure 7) after 48 hr fermentation. While ethanol production was 4.62 g/l or 0.045g/g DM in case of the combined TWS_{Enz}-TWS_F method. Thus UWS_{Enz}-TWS_F method proved to be best combination/technique and was adapted in the next experiments.

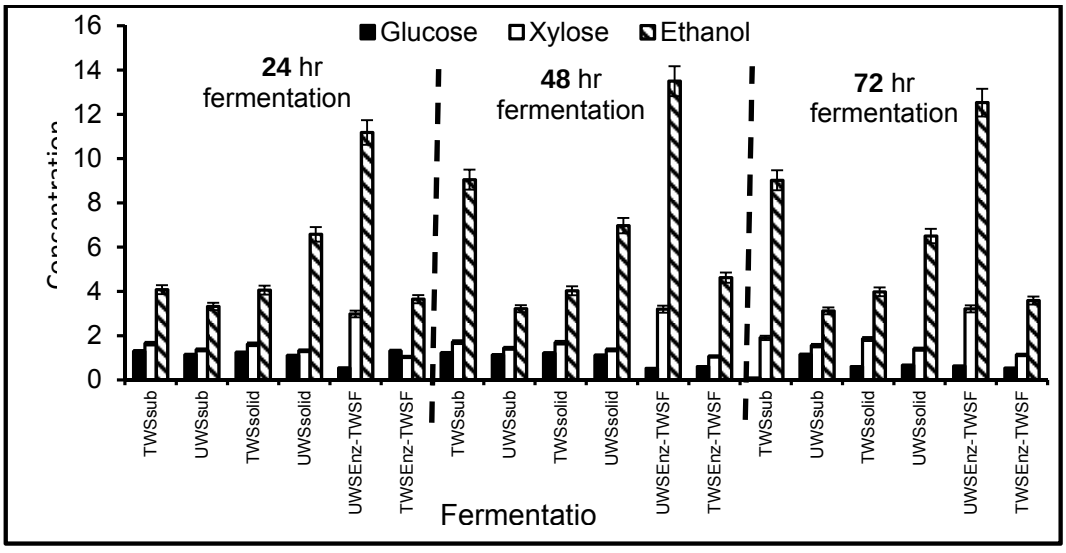

Figure 7: Study of different techniques for on-site production of cellulase by *T. reesei* $3EMS_{35}$ mutant and ethanol by *S. cerevisiae* in SVSF process for different time periods; 24 hr, 48 hr and 72 hr fermentation. Ethanol yield represented in terms of g/l of TWS.

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Using commercial enzyme, yield of ethanol upto 81 % have previously been reported (Chen et al., 2008). Our results of ethanol production are in agreement to the ones reported in literature (Angelidaki et al., 2009).

Different process combinations have been applied for improvement of ethanol production. (1) In separate hydrolysis and fermentation (SHF), the enzymatic hydrolysis and fermentation runs separately under own optimal conditions. SHF has a faster hydrolysis rate under optimum conditions as also shown in the study of Marques et al. (2008). (2) In simultaneous saccharification and fermentation process (SSF), saccharification and fermentation run simultaneously. The SSF process usually results in higher overall yields and shorter fermentation time (Tomas-Pejo et al., 2008). Temperature optima for yeast growth (37-38 °C) and enzymatic hydrolysis (50 °C) are different, which means that the conditions used in SSF cannot be optimal for both the hydrolysis and yeast fermentation (Ohgren et al., 2007; Wang et al., 2013). Alfani et al. (2000) compared SHF with SSF for bioconversion of steam-exploded wheat straw and reported that contrary to substantially faster ethanol productivity in SSF, the final ethanol yield was higher in SHF process. (3) The advantages of both SSF and SHF are combined in SVSF, which includes first enzymatic hydrolysis and then a subsequent fermentation phase in the same vessel. We brought further innovation and modification in SVSF process by integration of three important stages: on-site production of cellulase enzyme, hydrolysis of wheat straw, and fermentation into ethanol. This is a new technique and no evidence from literature is found. Results show that this new technique is promising, and economically advantageous because no external enzyme is needed and the hydrolytic process is conducted by the enzymes which are on-site produced by microorganisms.

Reactor experiments

Results from reactor experiments (Figure 8) show that most of cellulose was hydrolyzed within 24 hrs. Highest concentration of glucose (27.0 g/l) was achieved after 24 hr hydrolysis of 4 % TWS by on-site produced cellulase enzyme at 50 °C. In fermenter, highest titer of ethanol produced was 12.95 g/l (Figure 8).

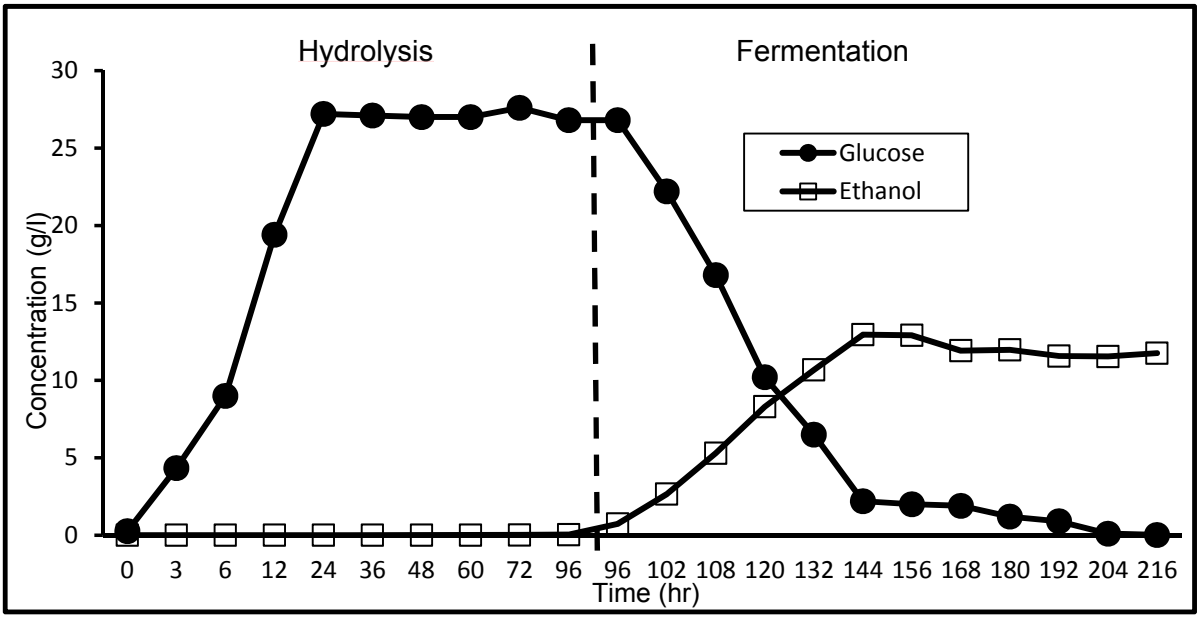

Figure 8: Production of ethanol in fermenter (3 litre); cellulase enzyme was produced on 1 % UWS by *T. reesei* $3EMS_{35}$ and this crude enzyme was used for hydrolysis of 4 % TWS (40 g/l) at 50 °C to produce glucose for ethanol production under anaerobic conditions by *S. cerevisiae*. Total working volume was 1000 ml.

The ethanol yield in fermenter (69.5 %) was lower than the yield in flasks (72 %) which could possibly be explained with the greater mechanical losses, and difficulties in maintenance of anaerobic conditions due to untight connection of pipes to fermenter and frequent samplings. We obtained 9.1 % less yield of ethanol than Chen et al., (2008) who achieved 81.1 % yield of ethanol in SSF of wheat straw pretreated with steam explosion and alkaline peroxide, possibly due to difference of technique of SSF. In our study ethanol production was 7 % higher than Zhua et al. (2006) who also did SSF of microwave assisted alkaline pretreated wheat straw and by *T. reesei* and *S. cerevisae* at 40 °C for 72 hrs and achieved 64.8 % yield of ethanol. Luo et al. (2011) used commercial enzymes and reported rate of hydrolysis of rapeseed straw slightly less (1 %) but rate of ethanol production 10 % more than us. In another study, Erdei et al. (2010) reported 15 % more ethanol yield from mixture of wheat straw and wheat meal in SSF using commercial cellulase enzymes and *S. cerevisiae*. No data on integration of process of, on-site cellulase production by locally mutagenised fungus using multi-agent mutagenesis (MAM) technique, and SVSF of acid pretreated wheat straw for ethanol production in fermenter is reported up until now; therefore novelty of our research work is confirmed.

CONCLUSIONS

In this study the fungus *T. reesei* $3EMS_{35}$ was exploited for cellulose production both in flask and fermenter experiments which was used for hydrolysis of pretreated wheat straw into glucose and then fermented to ethanol by *S. cerevisiae* PCSIR-12. The SEM and XRD techniques showed that structural modifications in the acid pretreated wheat straw were due to de-lignification. UWS was found to be a better substrate than TWS for on-site cellulase enzyme production. Lignin did not hinder the potential of fungus for cellulase enzyme production but acted as a booster. On-site cellulase enzyme production and SVSF are potential techniques for bioconversion of lignocelluloses to ethanol. Delignification and defibrillation of wheat straw due to acidic pretreatment released a large reactive area on the fiber surface, thus improving the accessibility of cellulase and bioconversion efficiency in SVSF. On-site production of cellulase and SVSF is a new technique and would probably reduce process costs. Use of whole crude broth of *T. reesei* resulted in cost reduction, improved saccharification (91 % of cellulose hydrolysed in 24 hrs) and enhanced bioethanol production (ethanol yield, 72 % in flask versus 69.5 % in fermenter).

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REFERENCES

Alfani F, Gallifuoco A, Saporosi A, Spera A, Cantarella M. Comparison of SHF and SSF processes for the bioconversion of steam-exploded wheat straw. J Ind Microbiol Biotech 2000;25:184–92.

Angelidaki I, Sanders W. Assessment of the anaerobic biodegradability of macropolutants. Rev Environ Sci Biotechnol 2004;3:117-29.

Angelidaki I, Lu X, Zhang Y. Optimization of H_2SO_4 catalysed hydrothermal pretreatment of rapeseed straw for bioconversion to ethanol: Focusing on pretreatment at high solids content. BioresourceTechnol 2009;100:3048-53.

Arantes V, Saddler JN. Access to cellulose limits the efficiency of enzymatic hydrolysis: the role of amorphogenesis. Biotechnol Biofuels 2010;3:4.

Atwell WA. An overview of wheat development, cultivation and production. Cereal Foods World 2001;46:59-62.

Barta Z, Kovacs K, Reczey K, Zacchi G. Process design and economics of on-site cellulase production on various carbon sources in a softwood-based ethanol plant. Enzyme Res 2010;2010:734182.

Cannella D, Hsieh CC, Felby C, Jørgensen H. Production and effect of aldonic acid during enzymatic hydrolysis of lignocellulose at high dry matter content. Biotechnol Biofuels 2012;5:26. doi:10.1186/1754-6834-5-26.

Çetinkol ÖP, Dibble DC, Cheng G, Kent MS, Knierim, B, Auer M et al. Understanding the impact of ionic liquid pretreatment on eucalyptus. Biofuels 2010;1:33-46.

Chartchalerm I, Tanawut T, Thikamporn K, Ponpitak P, Virapong P. Appropriate technology for the bioconversion of water hyacinth (Eichhornia crassipes) to liquid ethanol. Future prospects for community strengthening and sustainable development. EXCLI J 2007;6:167-76.

Chen H, Han Y, Xu J. Simultaneous saccharification and fermentation of steam exploded wheat straw pretreated with alkaline peroxide. Process Biochemistry 2008;43:1462-6.

Cullen D, Kersten PJ. Enzymology and molecular-biology of lignin degradation. In: Bramble R, Marzluf G (eds.): The Mycota III (pp 297-314). Berlin: Springer-Verlag, 1996.

Demirbas A. Bioethanol from cellulosic materials: a renewable motor fuel from biomass. Energy Sources 2005;27:327–37.

Detroy RW, Cunningham RL, Bothast RJ, Bagby MO, Herman A. Bioconversion of wheat straw cellulose/hemicellulose to ethanol by *Saccharomyces uvarum* and *Pachysolen tannophilus.* Biotechnol Bioeng 1982;24:1105-13.

Erdei B, Barta Z, Sipos B, Reczey K, Galbe M, Zacchi G. Ethanol production from mixture of wheat straw and wheat meal. Biotechnol Biofuel 2010; 3:16.

Gaensly F, Wille CF, Brand D, Bonfim BMT. Iron enriched *Saccharomyces cerevisiae* maintains its fermenting power and bakery properties, Cienc. Technol Aliment Compinas 2011;31:980-3.

Han L, Feng J, Zhang S, Ma Z, Wang Z, Zhang X. Alkali pretreatment of wheat straw and its enzymatic hydrolysis. Brazil J Microbiol 2012;43:53-61.

Hu J, Arantes V, Pribowo A, Saddler JN. The synergistic action of accessory enzymes enhances the hydrolytic potentialof a "cellulase mixture" but is highly substrate specific. Biotechnol Biofuels 2013;6: 112.

Ibbett R, Gaddipati S, Hill S, Tucker G. Structural reorganization of cellulose fibrils in hydrothermally deconstructed lignocellulosic biomass and relationships with enzyme digestibility. Biotechnol Biofuels 2013;6(1):33.

Idrees M, Adnan A, Shiekh S, Qureshi FA. Optimization of dilute acid pretreatment of water hyacinth biomass for enzymatic hydrolysis and ethanol production. EXCLI J 2013;12:30-40.

Karagöz P, Rocha IV, Özkan M, Angelidaki I. Alkaline peroxide pretreatment of rapeseed straw for enhanced bioethanol production by same vessel saccharification and co-fermentation. Bioresource Technol 2012;104:349-57.

Khokhar UZ, Syed Q, Nadeem M, Baig S, Irfan M, Gull I et al. Delignification of wheat straw with acid and hydro-steam under pressure. World Appl Sci J 2010;11:1524-30.

Kim TH, Lee YY. Fractionation of corn stover by hot-water and aqeous ammonia pretreatment. Bioresource Technol 2006;97:224-32.

Kongjan P, Min B, Angelidaki I. Biohydrogen production from xylose at extreme thermofilic temperatures (70 °C) by mixed culture fermentation. Water Res 2009;43:1414-24.

Kristensen JB, Thygesen LG, Felby C, Jørgensen H, Elder T. Cell-wall structural changes in wheat straw pretreated for bioethanol production. Biotechnol Biofuels 2008;1(1):5.

Kumar P, Barrett DM, Delwiche MJ, Strove P. Methods for pretreatment of lignocellulosic biomass for efficient hydrolysis and biofuel production. Industr Eng Chem Res 2009;48:3713-29.

Li C, Knierim B, Manisseri C, Scheller VH, Auer M, Vogel PK et al. Comparison of dilute acid and ionic liquid pretreatment of switch grass: Biomass recalcitrance, delignification and enzymatic saccharification. BioresourceTechnol 2010;101:4900-6.

Luo G, Talebinia F, Karakashev D, Xie L, Zhou Q, Angelidaki I. Enhanced bioenergy recovery from rapeseed plant in biorefinery concept. BioresourTechnol 2011;102:1433-9.

Marques S, Alves L, Rosero CJ, Girio MF. Conversion of recycled paper sludge to ethanol by SHF and SSF using *Pichiastipitis*. Biomass Biotechnol 2008; 32:400-6.

Mehmood S, Gulfraz M, Rana FN, Ahmad A, Ahring BK, Minhas N, Malik MF. Ethanol production from Sorghun bicolor using both separate and simultaneous saccharification and fermentation in batch and fed batch systems. Afr J Biotechnol 2009;8(12): 2857-65. doi:10.5897/AJB09.101.

More SS, Renuka PS, Pruthvi K, Swetha M, Malini S, Veena SM. Isolation, purification and characterization of fungal laccase from Pleurotus sp. Enzyme Res 2011;2011:248735. doi:10.4061/2011/248735.

Ohgren K, Vehmaanpera J, Siika M, Galbe M, Viikari L, Zacchi G. High temperature enzymatic prehydrolysis prior to simultaneous saccharification and fermentation of steam pretreated corn stover for ethanol production. Enz Microb Technol 2007;40: 607–13.

Reczey K, Szengyel Z, Eklund R, Zacchi G. Cellulase production by T. reesei. Biores Technol 1996; 57:25-30.

Saha BC, Iten LB, Cotta MA, Wu YV. Dilute acid pretreatment, enzymatic saccharification, and fermentation of rice hulls to ethanol. Biotechnol Prog 2005;21:816-22.

Saliu KB, Sani A. Bioethanol potentials of corn cob hydrolysed using cellulases of *Aspergillus niger* and *Penicillium decumbens*. EXCLI J 2012;11:468-79.

Segal L, Creely L, Martin AE, Conrad CM. An empirical method for estimating the degree of crystallinity of native cellulose using X-ray diffractometer. Text Res J 1959;29:786-94.

Shen J, Agblevor AF. Ethanol production of semi-simultaneous saccharification and fermentation from mixture of cotton gin waste and recycled paper sludge. Bioprocess Biosyst Eng 2011;34:33–43.

Socha MA, Plummer PS, Stavila V, Simmons AB, Sing S. Comparison of sugar contents for ionic liquid pretreated Douglas-fir woodchips and forestry residues. Biotechnol Biofuels 2013;6:61. doi:10.1186/1754-6834-6-61.

Sorensen A, Teller PJ, Lubeck PS, Ahring BK. On-site enzyme production during bioethanol production from biomass: Screening for suitable fungal strain. Appl Biochem Biotechnol 2011;164:1058-70.

Tao L, Chen X, Kuhn E, Tucker M, Aden A, Elander R et al. Improved ethanol yield and reduced minimum ethanol selling price by modefying low severity dilute acid pretreatment with acetylation and mechanical refining: 2) Techno-economic analysis. Biotechnol Biofuels 2012;5:69. doi:10.1186/1754-6834.

Tomas-Pejo E, Oliva JM, Ballesteros M, Olsson L. Comparison of SHF and SSF processes from steam-exploded wheat straw for ethanol production by xylose fermenting and robust glucose-fermenting *Saccharomyces cerevisiae* strains. Biotechnol Bioeng 2008;100:1122-31.

Tong M, Smith LH, McCarty PL. Methane fermentation of selected lignocellulosic materials. Biomass 1990;21:239–55.

Trajano HL, Engle NL, Foston M,Tschaplinski AJ, Wyman CE. The fate of lignin during hydrothermal pretreatment. Biotechnol Biofuels 2013;6:110. doi:10.1186/1754-6834.

Wang K, Yang H, Wang W, Sun CR. Structural evaluation and bioethanol production by simultaneous saccharification and fermentation with biodegraded triploid poplar. Biotechnol Biofuels 2013;6: 42. doi:10.1186/ 1754-6834-6-42.

Xiao LP, Sun Z J, Shi ZJ, Xu F, Sun RC. Impact of hot compressed water pretreatment on the structural changes of woody biomass for bioethanol production. BioResour 2011;6:1576-98.

Zhua S, Wu Y, Yu Z, Zhang X, Wang C, Yu F et al. Production of ethanol from microwave-assisted alkali pretreated wheat straw. Process Biochem 2006;41: 869-73.